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**REPORT No. J-262-3 (Progress Report)**

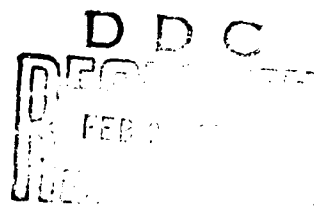
**CONTRACT No. DA-92-557-FEC-39509**

**BIOCHEMICAL STUDIES ON THE TOXIC NATURE OF SNAKE VENOM**

by

Chen-chung Yang, D. Med. Sc.

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December 1967

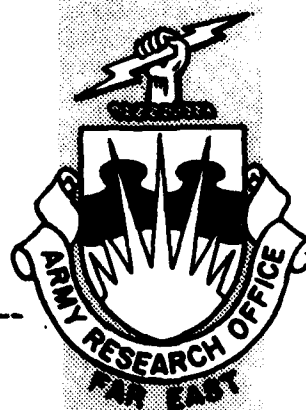
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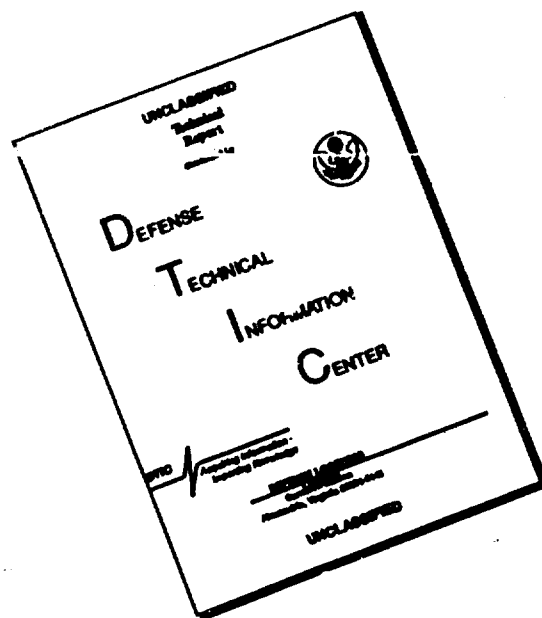
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DA Project/Task Area/Work Unit No. 2N014501B71D 00 063FE

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The crystalline toxin, cobrotoxin, has been obtained from Formosan cobra venom and was proved to be the main toxic protein in the venom. The present study was initiated to investigate the properties of the  $I^{131}$  labeled cobrotoxin and its distribution together with excretion after injection into rabbits.

#### 1. Iodination of cobrotoxin with $I^{131}$

Cobrotoxin was labeled with  $I^{131}$  according to the method of Hagli with slight modification as follows: 6.6 mg of the toxin in 1 ml of 1 M glycine buffer (pH 9.0) was injected into the labeling solution, prepared by adding 1 mc  $I^{131}$  to a maximum of 0.38 to 0.12 ml of the standard iodine monochloride solution. After 10 minutes, the free iodine was removed by passing through a Sephadex G-25 column (1 x 20 cm), and the effluent was collected every 1.5 ml. Radioactivity was counted with a well-type scintillation counter and protein was determined by the biuret method.

Under the conditions described, about 80 % iodine was incorporated into cobrotoxin. By chromatographic detection of free iodine, using Whatman No.1 filter paper and methanol, the  $R_f$  values of cobrotoxin and iodine were 0 and 0.5-0.6 respectively. The labeled cobrotoxin preparation was found to contain less than 1 % free iodine.

As shown in Fig. 1, the exhausted iodination of cobrotoxin with increasing amounts of  $ICl$  resulted in a maximum incorporation of 4 moles iodine per mole cobrotoxin. It has been reported that in iodination of some proteins, such as lactogenic hormone, zein and insulin, only tyrosine is iodinated. However, histidine can also be iodinated by the method.

Therefore, the possibility of iodination of amino acids other than tyrosine in cobrotoxin was studied. The labeled cobrotoxin, which had maximum iodination, i.e. 4 moles iodine per molecule toxin, was hydrolyzed with alkali in a sealed tube in the presence of tyrosine, moniodotyrosine and diiodotyrosine. After 20 hours hydrolysis at  $100^\circ C$ , paper chromatography and subsequent autoradiography were performed. The major spot on the autoradiogram was identified as diiodotyrosine, and two minor spots as moniodotyrosine and free iodine. However, not even the trace of iodinated histidine was found. It is likely that the small amounts of the moniodotyrosine and free iodine are given rise from the decomposition of diiodotyrosine, which resulted from the exhausted iodination of the cobrotoxin. Tyrosine residues in cobrotoxin were, therefore, estimated to be 2 moles per molecular weight of 6,800.

As shown in Fig. 2, the UV absorption curve of the labeled cobrotoxin was as changed as that of iodinated serum albumin. However, the lethality and antigenicity of the cobrotoxin remained unchanged even after exhausted iodination, suggesting that tyrosine was not involved in the active sites of the toxicity and antigenicity.

## 2. Excretion of $I^{131}$ labeled cobrotoxin in rabbit urine

Male rabbits weighting from 2 to 2.5 kg were given 50 ml of normal saline solution by stomach tube and injected intravenously with 25  $\mu$ g of the labeled cobrotoxin. Every 2 ml of urine was collected by catheterization and radioactivity of each fraction was counted. As shown in Fig. 3, about 70 % of the radioactivity was excreted in the urine within 5 hours. But iodinated serum albumin was excreted in amounts less than 10 % under the same condition.

The urine of the fraction No. 3, collected 20 minutes after injection, and of fraction No. 20, collected 4 hours after injection, were passed through a sephadex G-25 column. As shown in Fig. 4, the majority of the radioactivity in fraction No. 3 was in large molecule, while in the urine collected in fraction No. 20, the radioactivity appeared in two peaks of equal size, one large, the other a small molecule.

In order to investigate the properties of the radioactive compounds excreted in the urine, the following experiments were done. 0.01 ml of urine specimens collected 2 and 4 hours after injection were applied on Whatman No. 1 filter paper. After chromatography, the radioactive compounds were separated into two spots, one at the starting point as the labeled cobrotoxin; the other located as free iodine. In another experiment, 0.6 ml of urine collected between 13 and 20 minutes after injection of 800  $\mu$ g labeled cobrotoxin were injected into two mice. The mice were all died within two hours, while the injection of the same amount of urine collected before injection of the toxin did not kill mice. After electrophoresis of the same urine for 16 hours, the radioactive substance showed one band, slightly toward the cathode. This position was almost identical to that of the labeled cobrotoxin.

In the present experiment only a small amount of the toxin (25  $\mu$ g) was injected. The reason why the toxin was excreted so rapidly in urine is obscure at the present time. However, two reasons might be suggested: the toxin might freely pass through the glomerular filter without reabsorption from the tubule lumen, since the molecular weight is small; or, the toxin might have some toxic action in the kidney and effect excretion.

In order to know whether cobrotoxin has any toxic effect on the renal function, radiiodinated serum albumin was injected into rabbits with and without non-labeled cobrotoxin and urine was collected. The excretion of radiiodinated serum albumin in urine within 5 hours after injection was 5-6 %. Cobrotoxin does not affect the excretion significantly (Fig. 5). Analysis of creatinine, uric acid, sugar and total nitrogen in urine show no difference before and after injection of cobrotoxin. It seems that cobrotoxin does not affect the renal function in our experimental condition. Therefore, it is concluded that rapid excretion of the injected  $I^{131}$  labeled cobrotoxin is not likely due to the change of renal function by cobrotoxin.

### 3. Distribution of $I^{131}$ labeled cobrotoxin in immunized and non-immunized rabbits

Male rabbits weighing from 2.0 to 2.5 kg were immunized by injecting increasing doses of cobrotoxin with Freund's complete adjuvant. Six to 360  $\mu$ g per kg body weight were injected subcutaneously into the footpads at 6-day intervals during a period of two months. Six to 15 days after the last shot, the immunized rabbits were injected  $I^{131}$ -labeled cobrotoxin (500-750  $\mu$ g) intravenously.

The scanograms of the whole immunized rabbits after injection of the labeled toxin (500  $\mu$ g) were shown in Fig. 6. Radioactivity was first revealed in the region of the chest (Fig. 6a), 3 hours after injection, then gradually shifted in the abdominal region (Fig. 6b, c, d). On the contrary, in the non-immunized rabbit, the radioactivity was localized in the bladder 2 hours after injection of 40  $\mu$ g of the labeled toxin. (Fig. 7).

Five hours after injection, a rabbit was sacrificed and the radioactivity in each organ was measured. As seen from Table I, 66 % of the injected radioactivity was demonstrated in the liver, while, spleen has the highest  $I^{131}$ -count per gm tissue basis. It is obvious that  $I^{131}$  content in liver was observed on the chest region in the scanogram whereas  $I^{131}$  content in kidney and bladder was observed in the abdominal region. As shown in Table II, most of the radioactivity were found in the soluble fraction after cell fractionation. Small amounts found in microsomes, nucleus, and mitochondrial fractions may be due to the contamination of soluble fraction during fractionation.

The scanogram of rabbit 24 hours after injection (Fig. 6-d), showed radioactivity in the neck region, which demonstrated that deiodination took place in the immunized rabbit after injection of  $I^{131}$  labeled cobrotoxin.

The excretion of radioactive substances after injection of  $I^{131}$ -labeled toxin was much slower in the immunized rabbits than in non-immunized rabbit. In non-immunized rabbit, approximately 70 % of the injected radioactivity was excreted in the urine within 5 hours, while in the immunized rabbit, only 5-7 % of the injected radioactivity was excreted in 4 hours, and 60-70 % of the radioactivity was excreted within 4 days (Fig. 8).

$I^{131}$ -labeled serum protein have been used in the study of the fate of protein after injection into the immunized and non-immunized rabbits. It was demonstrated that the only trace amount of the labeled protein remained in blood for several hours after injection into the non-immunized rabbits, but the most of the  $I^{131}$ -labeled serum protein was removed from the blood soon after the injection to the immunized rabbits. It is interpreted that  $I^{131}$  labeled serum protein-antibody complex, which formed in the immunized animal is phagocited by the liver or spleen cells. Phagocytosis of radioiodinated serum



albumin-antibody complex was also found in mouse peritoneal cell. Apparent prolonged retention of the radioactivity after injection of  $I^{131}$ -labeled cobrotoxin in the immunized rabbits than in non-immunized rabbit was demonstrated in this experiment.

#### CONCLUSION

The exhausted iodination of cobrotoxin, the crystalline toxic protein obtained from Formosan cobra venom, with  $I^{131}$  resulted in the maximum incorporation of 4 moles iodine per mole toxin without changing its lethality or antigenicity. After intravenous injection of a sublethal dose of the labeled cobrotoxin into a rabbit, about 70 % of the radioactivity was excreted in urine within 5 hours. Most of the radioactivity excreted in the urine after 20 minutes injection was shown to be in the intact cobrotoxin fraction by gel filtration, paper electrophoresis and the toxicity test. In the urine collected 4 hours after injection, half of the radioactivity appeared in the free iodine fraction.

The scanogram of rabbits in both conditions, immunized and non-immunized, showed marked different pattern in distribution of radioactivity. In the immunized rabbit the radioactivity was first shown in the region of the chest then gradually shifted in the abdominal region. Sixty-six per cent of the injected radioactivity was demonstrated in the liver of the immunized rabbit, sacrificed 5 hours after injection of the labeled toxin. And most of the radioactivity was found in the soluble portion after cell fractionation. In the non-immunized rabbit the radioactivity was localized in the bladder 2 hours after injection. The excretion of the radioactivity after injection of  $I^{131}$ -cobrotoxin was much slower in the immunized rabbit than in the non-immunized rabbit.

Amino acid composition of cobrotoxin has been determined by the use of Technicon amino acid Autoanalyzer. As seen from Table III, cobrotoxin is composed of 15 kind of the common amino acids and is devoid of alanine, methionine and phenylalanine. Although a molecular weight of 11,000 was determined formerly by the method of Archibald for cobrotoxin, the minimal molecular weight calculated from amino acid analysis (61 residues of amino acids) is close to 6,800. It is noteworthy that with the exception of crotamin (Table III, last column) all the neurotoxins obtained from snake venoms contain 4 disulfide bridges and possess almost the same number of amino acids in the molecule.

The N-terminal amino acid was determined to be leucine by DNP- and PTC-methods. The C-terminal amino acid was identified as asparagine by the selective tritium-labelling procedure of Matsuo et al. and carboxypeptidase digestion.

The work on the amino acid sequence determination is in progress. The reduced and S-carboxymethylated toxin was digested with trypsin DCC (Diphenyl carbamyl chloride) or chymotrypsin in 0.1 N  $\text{NH}_4\text{HCO}_3$ , at pH 8.5. After 6 hours the reaction was stopped by freezing. The freeze-dried material was dissolved in a starting buffer solution and was loaded on a column (1.5 x 27 cm) of Dowex 50 X2. The column was developed with the buffer linearly increasing the pH and the ionic strength from 0.1 M pyridine-formate of pH 3.1 to 2.5 M pyridine-acetate of pH 5 by the aid of an Autograd consisting of 9 chambers. The flow rate was adjusted to 70 ml per hour and the appropriate tubes under the chromatographic peaks were pooled and lyophilized (Fig. 9).

Homogeneity of the peptide fractions obtained by column chromatography was examined by both paper chromatography and paper electrophoresis (Fig. 10). The heterogeneous fractions were further purified with 2 % pyridine acetate buffer at pH 5.4 by high voltage paper electrophoresis. The peptides finally obtained in homogeneous state were used for structure studies. Amino acid composition was determined on the Technicon amino acid Autoanalyzer, and the amino acid sequence of the peptides are being determined by the degradation procedure of Edman.

The positions of disulphide bridges in cobrotoxin are also being determined by a diagonal paper-electrophoretic technique. The crystalline toxin is digested with pepsin in 5 % formic acid. Cystine peptides are separated by paper electrophoresis and oxidized on paper by performic acid vapour. Electrophoresis at right angles to the first direction produces parallel groups of cysteic acid peptides lying off a diagonal. The fingerprint reveals the way in which the cysteic acid peptides were originally joined in the protein. The purified cysteic acid peptides are eluted for composition and sequence analysis.

We are in hope that the complete sequence of amino acid as well as the positions of disulphide bridges in cobrotoxin will be established during next year.

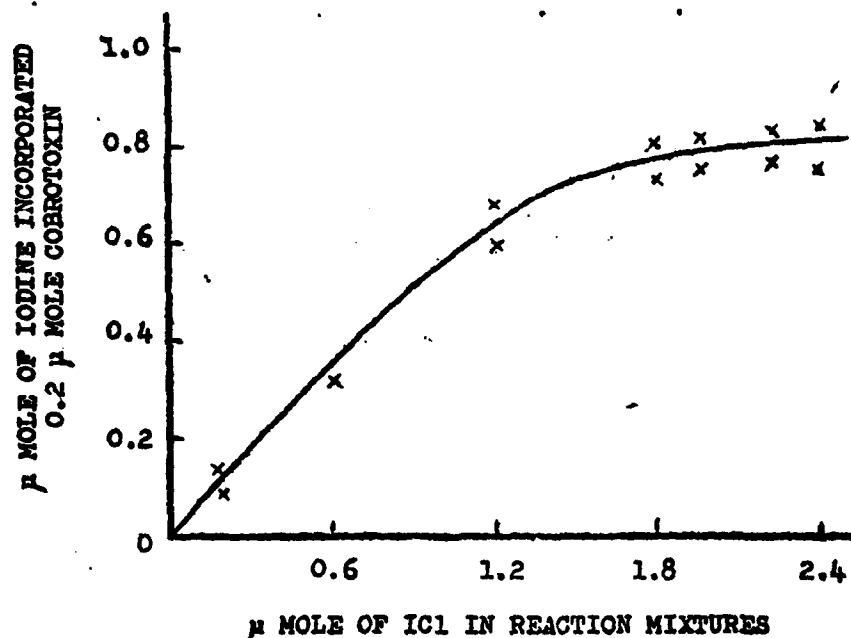


Fig. 1. Exhausted iodination of cobrotoxin with various amounts of ICl solution. Reaction was carried out at  $25 \pm 2^\circ$  for 20 min, in 1.5 ml glycine buffer (pH 9), containing 0.2  $\mu$  mole of cobrotoxin, 1  $\mu$ c  $I^{125}$ , and various amounts of ICl solution. One ml aliquot was then passed through a Sephadex G 25 column (1.1 x 20) and eluted with water. Every 1.5 ml of the effluent was collected by gravity. About 92 to 95 per cent of protein was obtained in fractions Nos. 5-8. Percentage of incorporation was estimated by comparing the radioactivity before and after passing through the column.

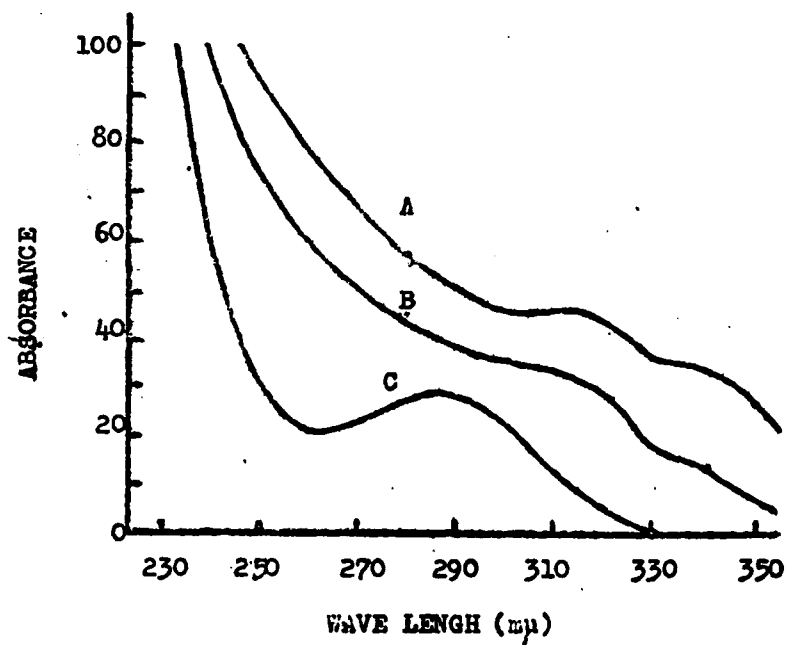
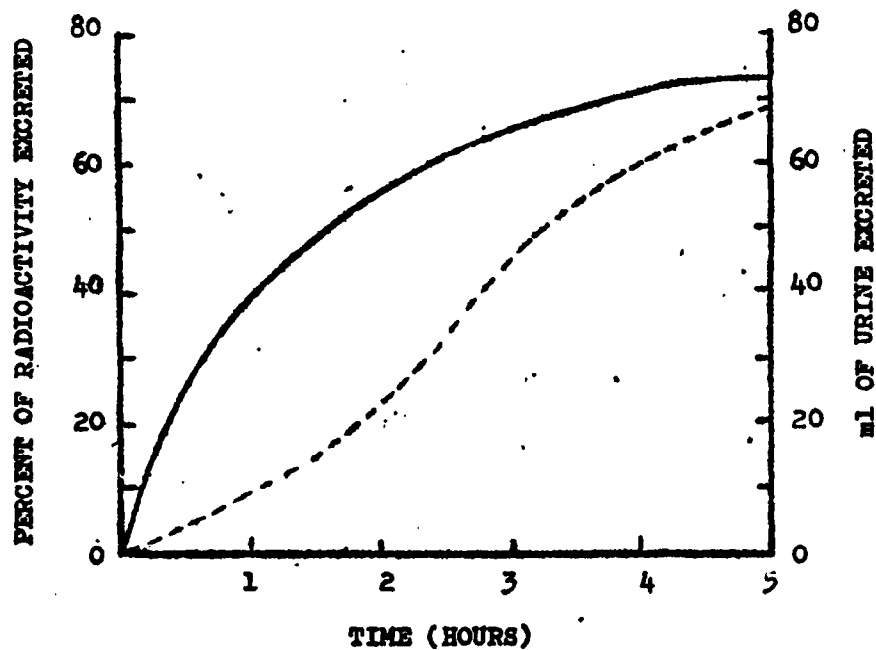


Fig. 2. Ultra-violet absorption curve of iodinated cobrotoxin and cobrotoxin plotted with a Beckman DK-2 spectrophotometer.  
 (a) Iodinated cobrotoxin, 4 moles iodine/mole toxin.  
 (b) Iodinated cobrotoxin, 2 mole iodine/mole toxin.  
 (c) Unlabeled cobrotoxin.



**Fig. 3.** Excretion of Radioactivity in urine after injection of  $^{131}\text{I}$  labeled cobrotoxin. Urine was collected from male rabbit by catheterization immediately after injection of 25  $\mu\text{g}$  toxin with 5  $\mu\text{c}$  radioactivity.  
 — : radioactivity excreted.  
 ----- : ml of urine excreted.

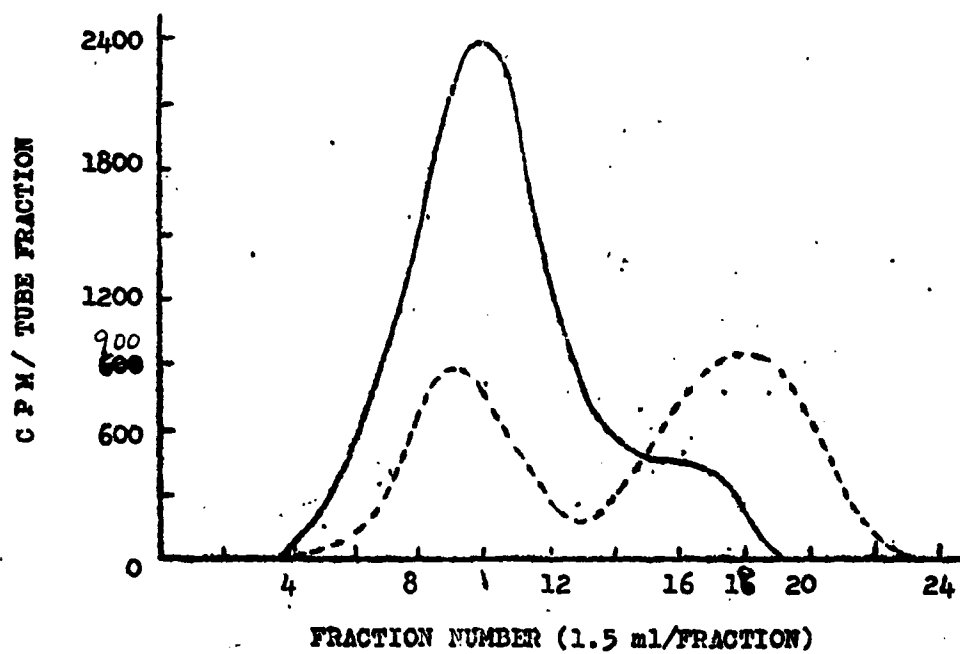


Fig. 4. Sephadex G 25 column chromatography of urine. 1 ml of the urine was applied to a Sephadex G 25 column (1.1 x 20 cm) and then eluted with water.  
 —: urine of fraction No. 3.  
 -----: urine of fraction No. 20.

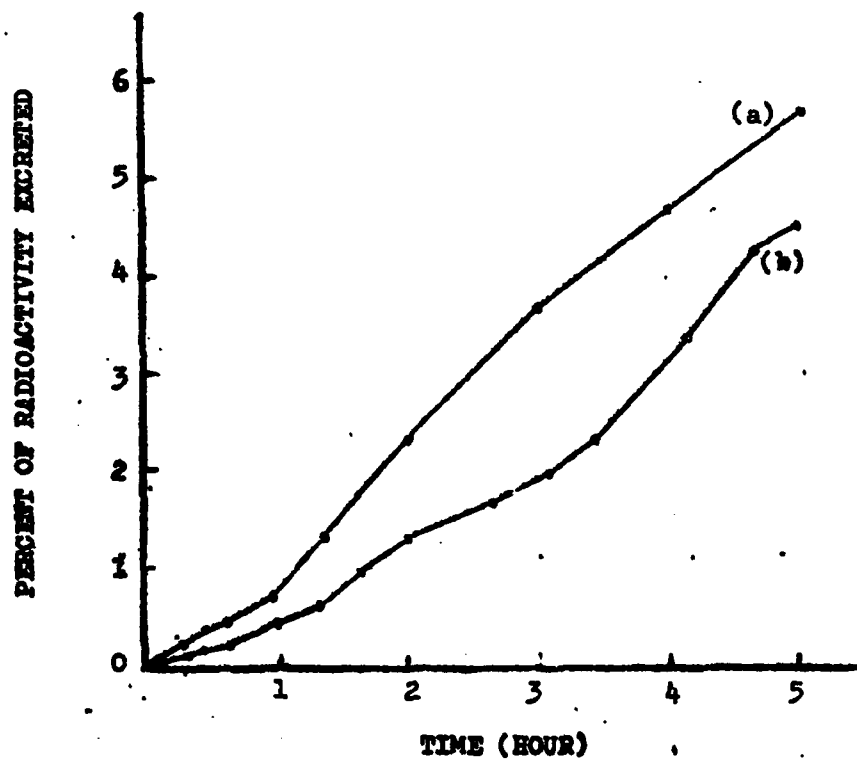


Fig. 5. Excretion of radioactivity in urine after injection of 2.6 mg radiolodinated serum albumin with (b) or without (a) cobrotoxin (30 ug).

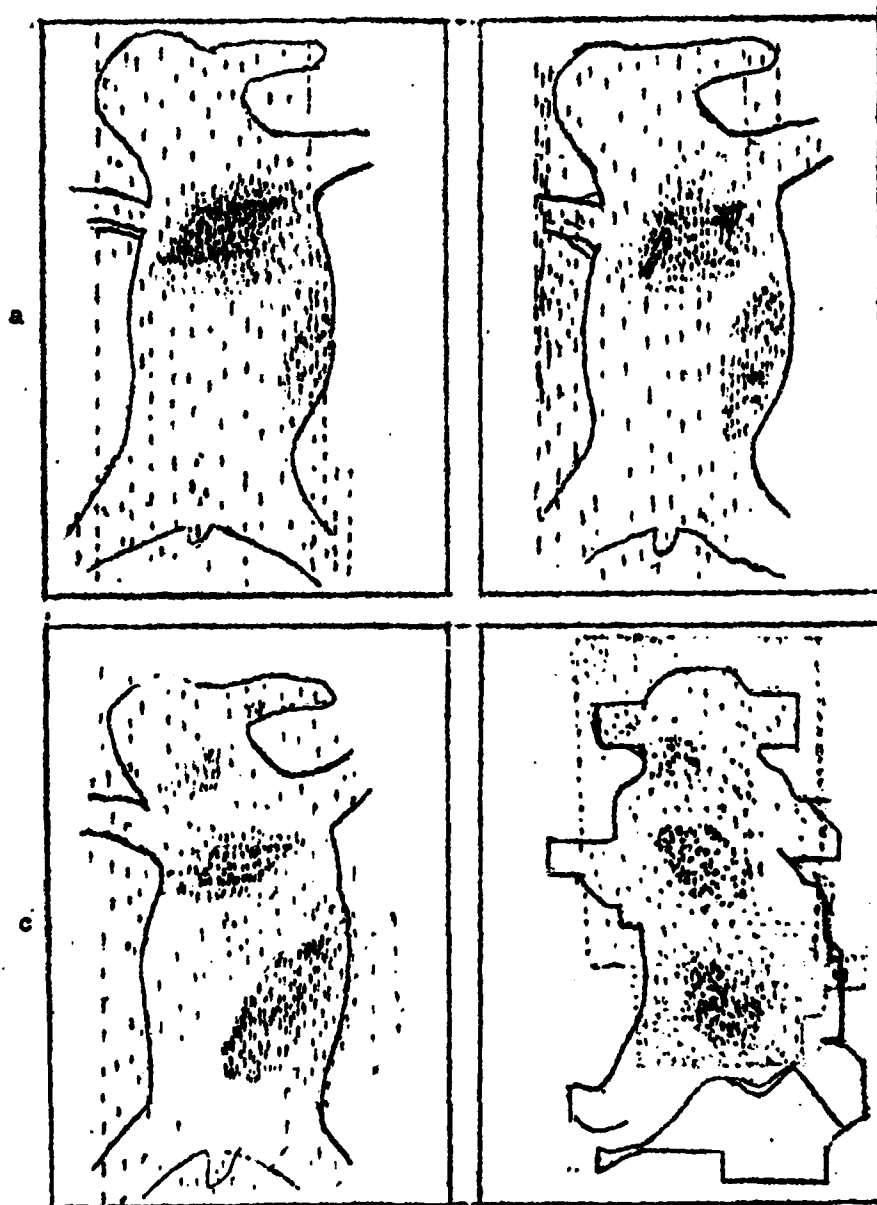


Fig. 6. Scanogram of immunized rabbit after injection of labeled toxin.  
 500  $\mu$ g of labeled cobrotoxin was injected into 2 kg rabbit and scanograms were taken: a, 3 hours; b, 5 hours; c, 8 hours; d, 24 hours after injection.



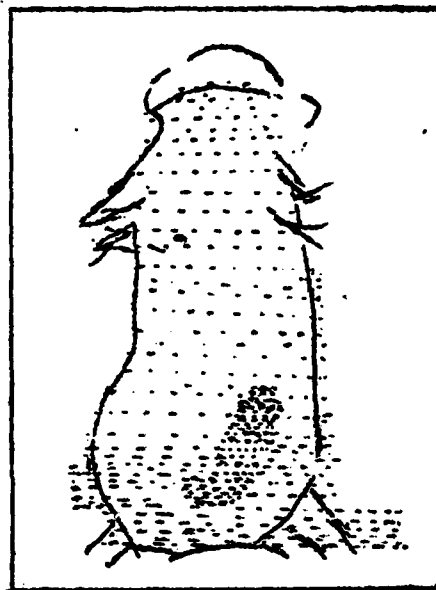


Fig. 7. Scanogram of non-immunized rabbit after injection of labeled cobrotoxin. 40  $\mu$ g of labeled cobrotoxin was injected into 2.5 kg of rabbit and scanogram was taken after 2 hours.

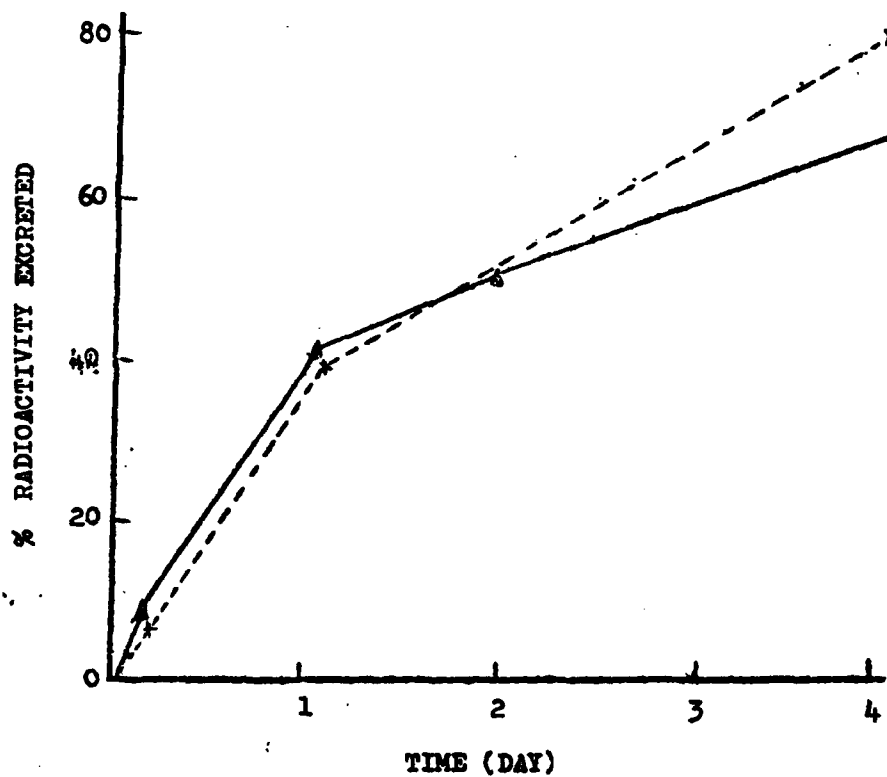


Fig. 8. Excretion of radioactivity in urine of immunized rabbits after injection of  $I^{131}$  labeled cobrotoxin.

(a) Immunized rabbit (2 kg), injected with 500  $\mu$ g of  $I^{131}$  labeled cobrotoxin.

(b) Immunized rabbit (2.2 kg), injected with 700  $\mu$ g of  $I^{131}$  labeled cobrotoxin.

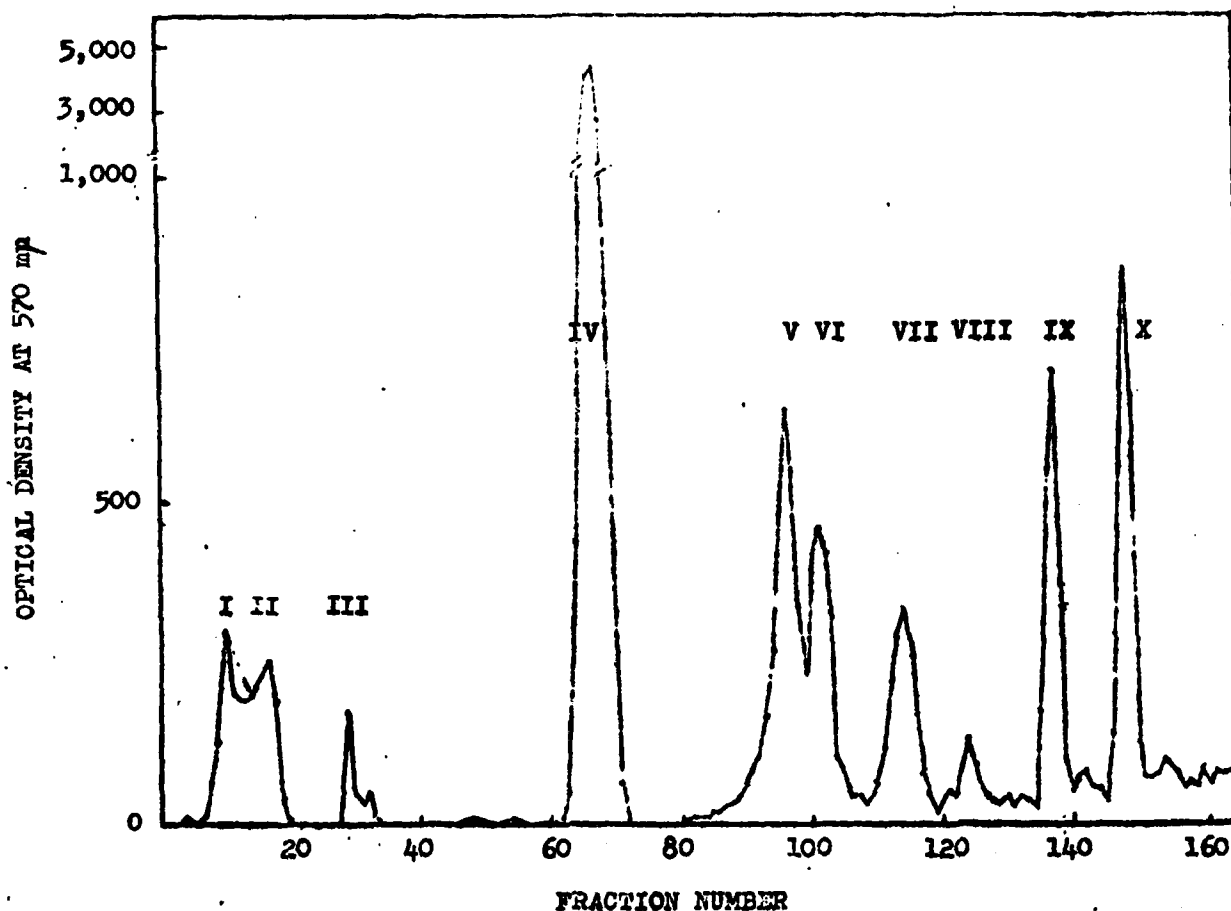


Fig. 9. Chromatographic fractionation of the peptides obtained by the tryptic hydrolysis of CM-cobrotoxin.

The peptides from 120 mg of the protein were loaded on a column (1.5 x 27 cm) of Dowex 50 x 2 (200-400 mesh), which was equilibrated with 0.1 M pyridine-formate buffer, pH 3.25. The column was developed with the buffer linearly increasing the pH and the ionic strength from 0.1 M pyridine-formate of pH 3.1 to 2.5 M pyridine-acetate of pH 5 by the aid of an Autograd consisting of 9 chambers. The flow rate was adjusted to 70 ml per hour and the effluent was collected in 5.5 ml fractions. Aliquots from each tube were analyzed by the ninhydrin method.

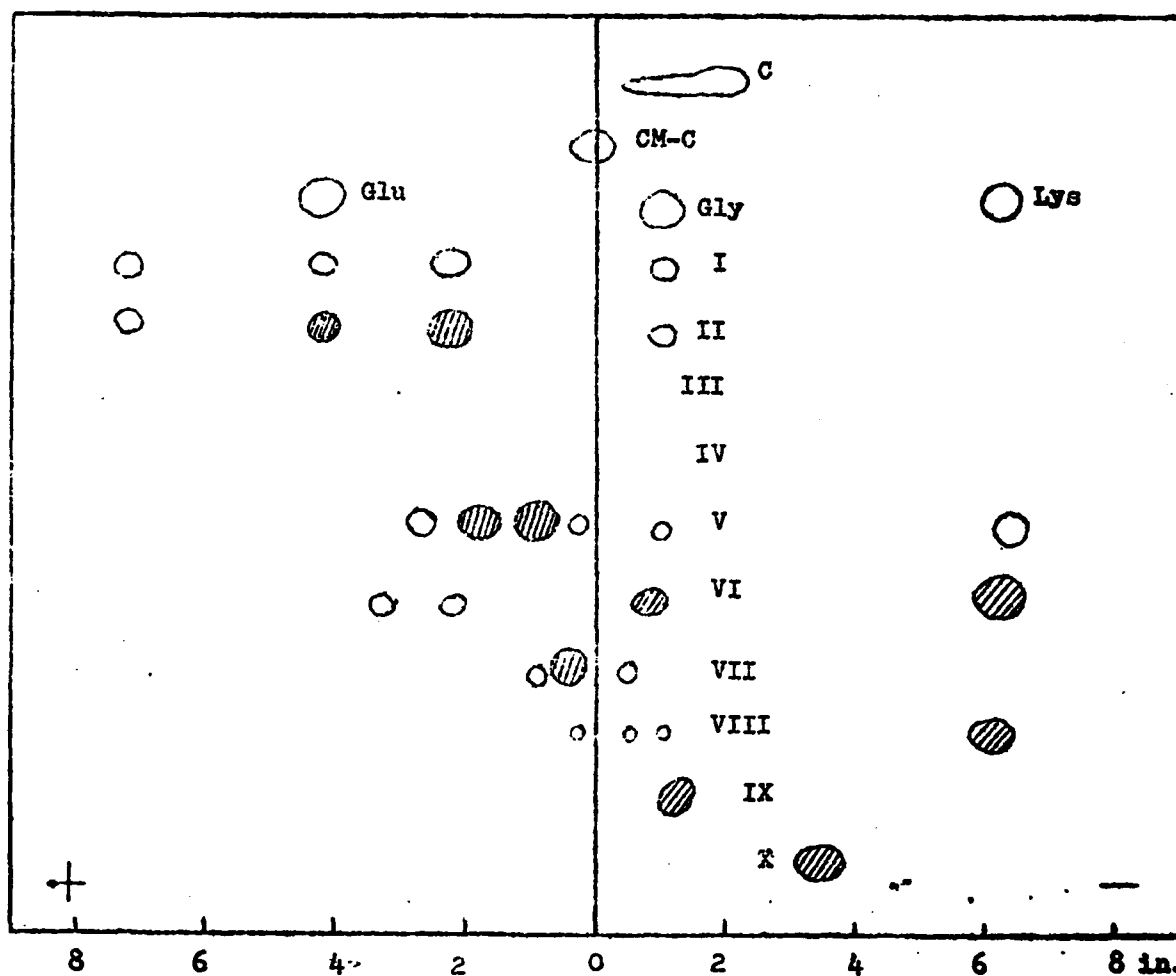


Fig. 10. Paper electrophoregram of peptide fractions obtained by the column chromatography (Fig. 9).

The electrophoresis was carried out at 2500 volts per 36 in. length of paper for 60 minutes with pyridine-acetic acid-water (7:2:490, v/v) of pH 5.4 as the electrolyte.

Table I  
Distribution of radioactivity in various organs of an  
immunized rabbits after intravenous injection of  
 $I^{131}$  labeled cohrotxin

Organ	Wet weight (gm)	CPM/organ	CPM/gm	% Radioactivity distribution
Spleen	1.2	$8.2 \times 10^4$	$6.8 \times 10^4$	2.1
Liver	48	$2.6 \times 10^6$	$6.1 \times 10^4$	66.6
Bladder*	47	$3.6 \times 10^5$	$7.5 \times 10^3$	9.3
Kidney**	15	$9.3 \times 10^4$	$6.2 \times 10^3$	2.4
Heart	5	$1.3 \times 10^4$	$2.6 \times 10^3$	0.33
Lymph node	2	$6.2 \times 10^3$	$3.1 \times 10^3$	0.15
Total				80.88

\* including urine

\*\* including blood

Table II

Distribution of radioactivity in subcellular fractions of liver and spleen of an immunized rabbit after intravenous injection of 720  $\mu\text{C}$  of  $\text{I}^{131}$  labeled cobrotoxin

subcellular fraction	% Distribution of radioactivity	
	liver	spleen
Supernatant	73	61
Microsome	13	12
Mitochondria	8	18
Nucleus	6	9

Table III

Amino acid composition of the neurotoxins isolated from snake venoms

	Naja naja atra (cobro- toxin)	Naja nigricollis (toxin $\alpha$ )	Laticauda semifasciata (erabutoxin)		Latico- toxin a	Androctonus australis (neurotoxin)		Crotalus terrificus (crotamin)
			a	b		I	II	
Aspartic acid	7	7	5	4	9	9	8	3
Threonine	7	8	5	5	4	2	3	
Serine	4	2	7	7	5	6	2	3
Glutamic acid	7	6	8	8	7		4	2
Proline	3	5	4	4	5	6	3	4
Glycine	7	5	5	5	5	6	7	5
Alanine						1	3	
Half-cystine	8	8	8	8	8	8	8	4
Valine	2	2	2	2	1	4	4	
Methionine								1
Isoleucine	2	3	4	4	2	3	1	1
Leucine	1	2	1	1	1	4	2	1
Tyrosine	2	1	1	1	1	3	7	1
Phenylalanine			2	2	1	1	1	2
Lysine	3	6	4	4	4	6	5	11
Histidine	2	2	1	2	2	1	2	3
Arginine	5	3	3	3	5	2	3	2
Tryptophan	1	1	1	1	1	1	1	3
Total	61	61	61	61	61	63	64	46
Min. mol. wt.	6785	6787	6760	6780	6880	6822	7249	5450

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